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Regulation of Mammary Gland Development by Tissue Interaction[1]

Gertraud W. Robinson,[2,4] A. B. Carmen Karpf,[3] and Klaus Kratochwil[3]

Development of the mammary glands is initiated in the embryo but the major part of their development occurs in the adult. While development in puberty and pregnancy is dependent on hormones, prenatal and early postnatal development appear to progress autonomously. Mutual and reciprocal epithelial-mesenchymal interactions are critical for both phases of development. Specific steps such as the formation of the bud, the first appearance of hormone receptors, formation of the primary sprout and ductal elongation have been shown to be governed by epithelial-mesenchymal signaling. In recent years, some of the signaling molecules that are required in these processes have been identified through gene inactivation. We discuss the potential role of these factors in mediating growth and differentiation. In addition we provide evidence that mammary epithelial cells from late embryonic stages are already capable of synthesizing milk proteins when subjected to appropriate hormonal stimulation.

KEY WORDS: Tissue interaction; steroid receptors; LEF-1; PTHrP; activin; milk proteins.

INTRODUCTION

The mammary gland is distinct from most other organs in that the major part of its development takes place during postnatal life. The primordia of the gland form early in embryogenesis as derivatives of the epidermis, but growth of the gland proceeds rather slowly until puberty when the parenchyma starts to extend and fill the mammary fat pad. Functional differentiation is reached only during pregnancy with the development of alveoli and the synthesis of specific milk proteins late in pregnancy and during lactation. Full functional activity commences around parturition when the inhibitory effects of progesterone are removed. The dependence of mammary development during puberty and pregnancy on regulation by reproductive hormones is well accepted. In contrast, the initiation of gland development and early morphogenesis occur independently of hormonal regulation and are governed by close-range interactions between epithelium and surrounding mesenchyme as in all epithelial-mesenchymal organs studied so far. However, one of the characteristics of mammary gland development is the interplay between regulation by systemic hormones and short-range tissue interactions: In the embryonic period, hormone responses both depend on, and interfere with epithelial-mesenchymal interactions, and in the adult gland, hormone-induced developmental steps appear to be mediated by epithelial-stromal interactions.

Here we review evidence for the role of tissue interaction throughout mammary development. Mutual interactions between epithelium and stroma regulate the formation of the primordial bud, its growth and the development of hormone responsiveness. Recent data from gene deletion experiments that affect the

[1] This review is dedicated to the memory of Clifford Grobstein whose conceptual and experimental contributions pioneered the study of tissue interaction in organogenesis.

[2] Laboratory of Genetics and Physiology, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bldg. 8, Rm. 101, Bethesda, Maryland 20982-0822.

[3] Institute of Molecular Biology, Austrian Academy of Sciences, Salzburg, Austria.

[4] To whom correspondence should be addressed. e-mail: gertraur@bdg10.niddk.nih.gov

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development of epithelial-mesenchymal organs provide clues to some of the signaling mechanisms that are common to the formation of skin appendages and of related structures such as teeth.

FORMATION OF THE MAMMARY RUDIMENT

The first morphological indication of mammary development is a localized thickening of the ectoderm or epidermis. In many mammals, the formation of the individual gland buds is preceded by the elevation of an epidermal "mammary crest" and a milk line that runs along most of the length of the trunk. This milk line then fragments and regresses except for the individual gland buds that may form (anteriorly, in the thoracic region as in primates and elephants, posteriorly in the inguinal region as in most ungulates, or all along the trunk as in pigs). No milk line appears in the mouse embryo where 5 pairs of epithelial buds arise separately (3 in the thoracic, 2 in the inguinal region) along an imaginary line running just ventral of the limbs.

In the mouse embryo, mammary buds are formed between embryonic day 10 (E10) and E11. The size of the bud slowly increases until a circumscribed epithelial ball is formed within the epidermis by E13. During this period, relatively few DNA-synthesizing cells are observed in the mammary epithelium and growth occurs mainly through accretion of cells from the adjoining epidermis (Fig. 1A; 1). Additional cells move in from the epidermis to form the "stalk" which connects the gland bud to the epidermis, thereby creating the characteristic "light bulb" shape of the E14 rudiment. During this time, the gland epithelium proper passes through an approximately 24 hour period of almost complete proliferative arrest, as seen by ^{3}H-TdR-incorporation (Fig. 1B).

A temporary cessation of DNA synthesis is also seen at comparable stages in the development of other skin derivatives, such as hair, whiskers, and feathers (2, 3). In the mammary bud, cell proliferation resumes at E14.5 but a primary sprout does not grow out before E16 (Figs. 1 and 2). Continued proliferation until the end of gestation leads to formation of a small ductal tree that consists of 10 to 15 branches arising from the single duct that emanates from the nipple. Mammary rudiments explanted *in vitro* follow the same time course, indicating that this early development, including outgrowth of the primary sprout, is determined by an intrinsic program and not by systemic factors (4).

At the time of bud formation, the mesenchyme (as the embryonic stroma is called) underlying the mammary bud appears in no way distinct from the rest of the dermis. In contrast to the almost instantaneous formation of dermal papillae under hair and whisker pegs, the mesenchymal cells bordering the mammary buds orient themselves only slowly around the epithelium. By E14, the epithelial gland buds are surrounded by a slightly denser mesenchyme consisting of several concentric layers of fibroblasts oriented around the epithelium. They are not set apart from the more distant dermis as is the case, for example, in the salivary gland. However, this "primary mammary mesenchyme" is distinguished by elevated RNA synthesis and the expression of specific genes. Upon formation of the primary sprout the epithelium pushes through this sheath of mesenchymal cells and penetrates the future fat pad, a cluster of preadipocytes in the deeper dermis that arises independently of the mammary epithelium, but becomes the "secondary" stroma of the gland supporting ductal morphogenesis in the late fetal and the entire postnatal period (Fig. 2).

These two types of mesenchyme display clearly distinct effects on growth and morphogenesis of various embryonic and adult epithelia as demonstrated in extensive studies by Teruyo Sakakura and colleagues. Primary mammary mesenchyme induces hyperplastic growth in experimentally associated E17 mammary epithelium and, when grafted into the fat pad of young virgin mice, even in adult mammary glands. (5). Mesenchyme from the prospective fat pad of E14 mice is able to support organotypic development of E14 salivary gland, glandular stomach, intestine and colon and 0E12 pituitary gland and pancreas. Mesenchyme from the fat pad of older stages gradually loses this capacity (6). Although the basis for these differences is presently unknown these observations may indicate a developmentally regulated expression of growth factors and trophic signals in these mesenchymal cells.

EARLY GENE EXPRESSION

Although the formation of the milk line along a precise dorso-ventral level, and the positioning of the individual buds along the anterior-posterior represent an extremely interesting instance of "pattern formation", we are not aware of any studies focusing on this aspect. There is also not much information on specific gene expression during formation of the mammary buds (E11) as most studies on the embryonic gland have been done at later stages. The first gene known

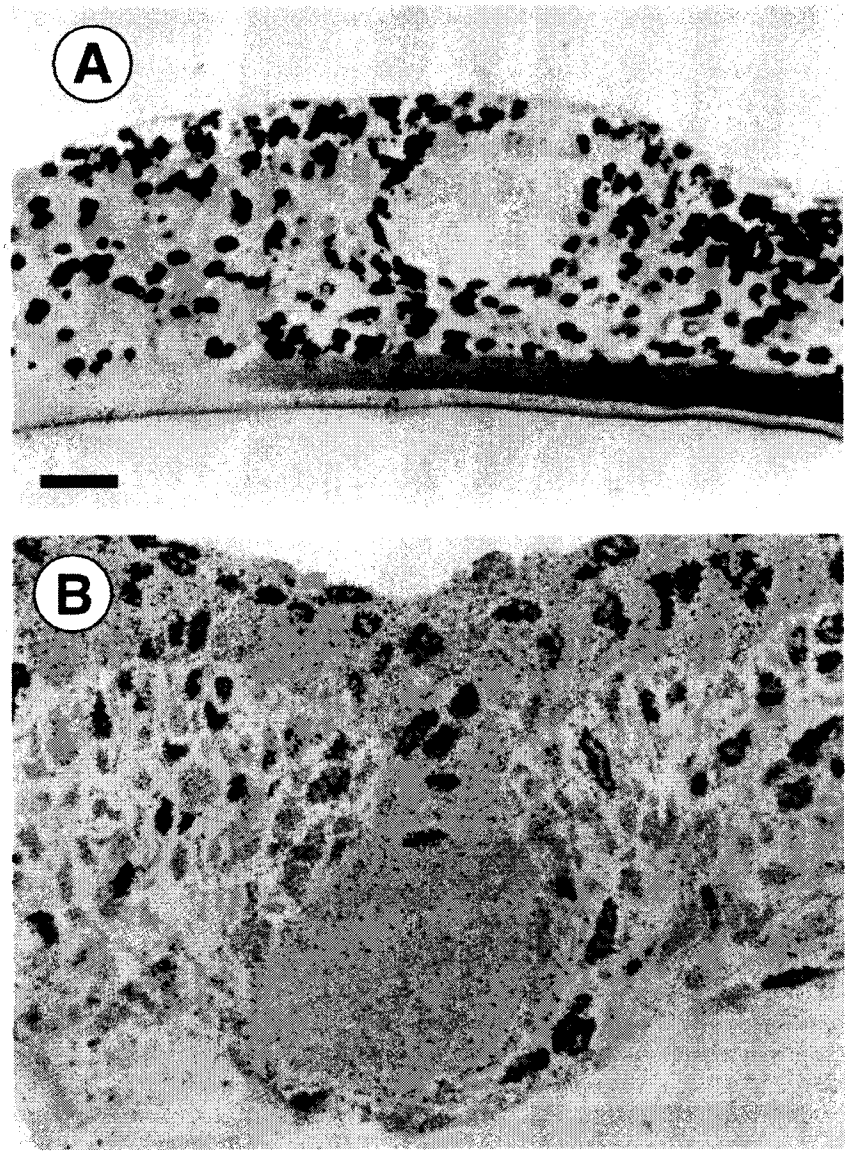

Fig. 1. Cell proliferation in 13 day embryonic mammary glands. Pieces of embryonic skin with attached mammary glands were allowed to incorporate ^{3}H-desoxythymidine for 1 hour and fixed immediately (A) or after a chase of 24 hours (B). (A) DNA synthesis, as evidenced by accumulation of silver grains over the nuclei of proliferating cells is absent in the epithelium proper. (B) Few proliferating cells are seen in the neck of the gland. Most likely these are cells that migrated from the epidermis into the gland proper during the chase period. Bar: 200μm (A), 50μm (B).

to be expressed specifically in the epidermis at the site of mammary gland formation is *Lef1*, a gene encoding a transcription factor of the HMG-box family. *Lef1* is activated during the formation of the mammary buds at E11.0, even before they become morphologically distinct. Interestingly, *Lef1* is expressed in all epidermal thickenings that initiate development of skin appendages including whisker, hair and (ectodemally derived) teeth, but its expression is not restricted to the epidermal compartment (7, 8).

By E12, the mammary epithelium has begun to express PTHrP (parathyroid hormone-related peptide). Expression continues throughout embryonic development but no information is available on the earliest appearance of these transcripts (9). At E13/14, the epithelial bud expresses the transcription factor genes

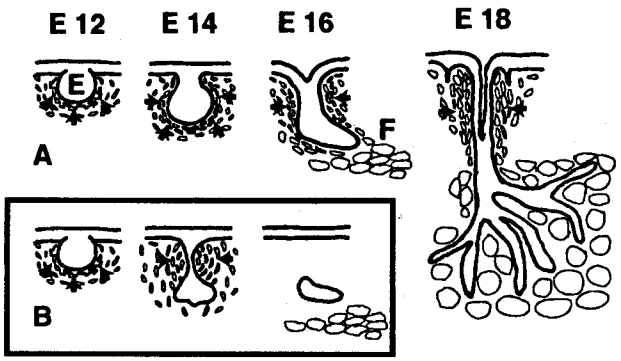

Fig. 2. Development of the embryonic mouse mammary gland. A small epithelial bud surrounded by primary mammary mesenchymal cells (arrowheads) is seen on E12 in both sexes. The size and shape of the epithelial bud in female embryos changes only slowly until E14. In male embryos, testosterone causes condensation of the mesenchyme around the neck of the gland and destruction of the mammary bud (inset B). Outgrowth of the primary sprout into the fat pad (F) and ductal morphogenesis starts around E16. A small ductal tree is seen at E18.

Msx1, *Msx2*, and *Lmx1b* (10, 11; R. Maas and R. Johnson, personal communication).

As mentioned earlier, the mesenchymal cells surrounding the growing mammary bud are distinguished from the dermal mesenchyme by elevated transcriptional and synthetic activities as evidenced by ^{3}H-uridine, ^{3}H-leucine and ^{3}H-glucosamine incorporation (Fig. 3). Furthermore, these cells are embedded in an extracellular matrix that is richer in heparan sulfates than the rest of the dermis as demonstrated by histochemistry (Kronberger and Kratochwil, unpublished observation). This mesenchyme is also characterized by the (elevated) transcription of specific genes, among them those coding for androgen and estrogen receptors (12, 13), for PPT-A (preprotachykinin, the common precursor for the potential signaling molecules substance P, neurokinin A, and neuropeptides K and γ) and its processing enzyme NEP (neutral endopeptidase; 14). Transcription of both steroid receptor genes and PPT-A is already elevated by E12. Furthermore, the primary mammary mesenchyme expresses the receptor for PTH/PTHrP (9), the secreted signaling protein BMP-4 (10), and the extracellular matrix components tenascin-C (15) and syndecan-1 (M. Bernfield, personal communication). At a slightly later stage, *Msx2* expression is also seen in mesenchymal cells (R. Maas, personal communication). *In situ* hybridization also showed elevated levels of *Fgf7* (*Kgf*) transcripts in the mesenchyme surrounding the epithelial bud (16). The expression domains of all these genes overlap remarkably and, together, they appear to define a specialized population of cells constituting the "primary mammary mesenchyme".

EARLY EPITHELIAL-MESENCHYMAL INTERACTIONS

Although tissue combination experiments have produced unequivocal evidence for multiple and reciprocal epithelial-mesenchymal interactions in the embryonic mammary gland, they do not provide evidence that shows decisively which of the two tissues is the initiator of mammary development. Neither has this problem been resolved for other skin appendages, not even for the most thoroughly studied system, the tooth (17). Tissue recombination experiments in the rabbit embryo, where the milk line appears on E13, suggest a leading role for the mesenchyme. At E12, i.e., before visible mammary differentiation, combinations of mesenchyme from the (prospective) mammary region with heterotopic epidermis of the head or neck yielded mammary structures whereas reciprocal combinations (of prospective mammary epidermis with nonmammary mesenchyme) did not. By E13, however, even just before the appearance of the milk line, "mammary epidermis" did form buds in association with mesenchyme from other regions (1). While this early study applied only morphological criteria for mammary development, Cunha *et al.* (18) later showed that E13 (mouse) mesenchyme was able to induce rat midventral or dorsal epidermis to form functional mammary epithelium as determined by immunohistochemical detection of casein and α-lactalbumin. Though these studies clearly show that the primary mammary mesenchyme can induce true mammary development in the epidermis, they do not address the question of the initiation of mammary development. Conceivably, even the "inducing" mammary mesenchyme of the E12 rabbit embryo may already have received decisive cues from the overlying ectoderm as recently shown for the initiation of limb development (19, 20).

While the processes initiating mammary development still remain unclear, there is fairly good evidence that formation of the primary mammary mesenchyme is governed by signals coming from the epithelial bud. When isolated epithelial gland buds are placed on mesenchyme that had not previously been in contact with a mammary epithelium, they induce the same mesenchymal responses including elevated ^{3}H-uridine and ^{3}H-glucosamine incorporation (Fig. 3), elevated production of tenascin-C (15), and the synthesis of andro-

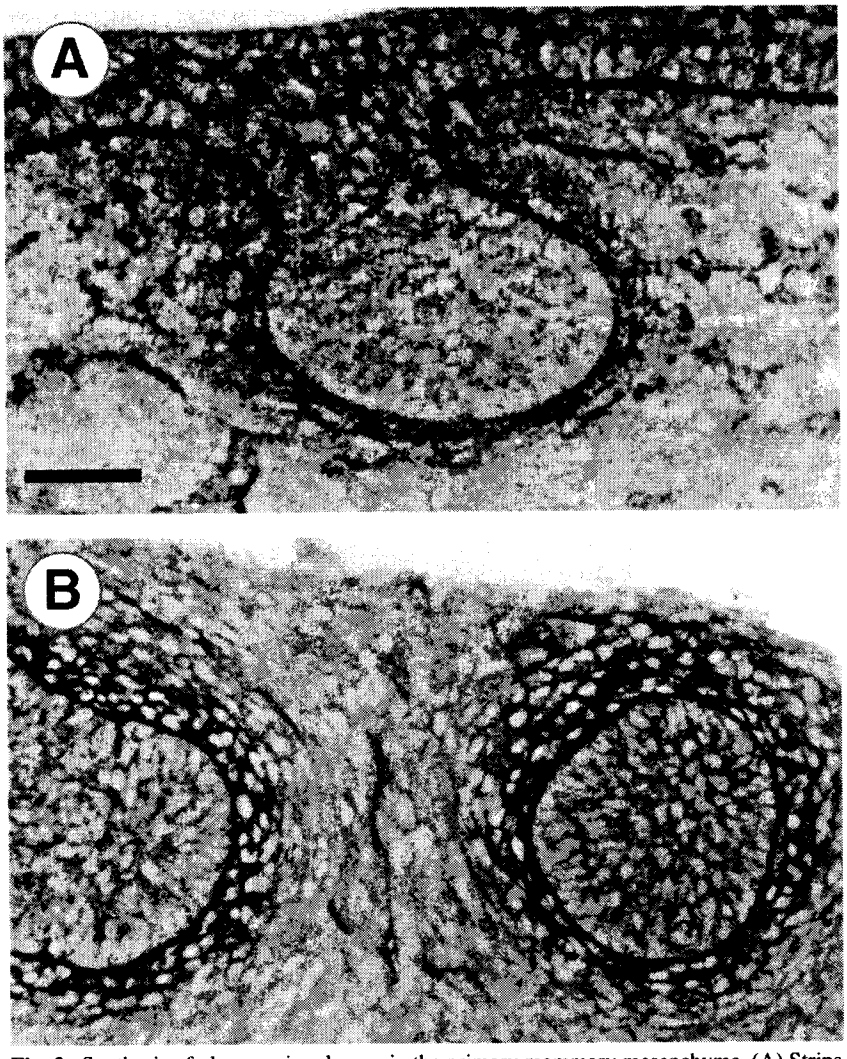

Fig. 3. Synthesis of glycosaminoglycans in the primary mammary mesenchyme. (A) Strips of ventral skin containing mammary glands were placed into short term culture and labeled with ^{3}H-glucosamine-containing medium for 12 hours and processed for radioautography. Elevated levels of ^{3}H-glucosamine incorporation are seen at the epithelial mesenchymal interface (basement membrane) and in the mesenchyme in the vicinity of the mammary epithelium. (B) Isolated mammary epithelia were placed on dorsal mesenchyme and grown in organ culture. After 3 days they were labeled as described earlier. The epithelia are surrounded by a halo of mesenchymal cells that show robust deposition of ^{3}H-glucosamine in the extracellular matrix. Bar; 200 μm (A), 250 μm (B).

gen and estrogen receptors (12, 13). The induction of steroid receptors was shown to be a specific property of mammary epithelium, not shared by other epithelia (epidermis, salivary gland, pancreas). Moreover, mesenchymal cells experimentally associated with mammary epithelium not only express androgen receptors but are also capable of executing the androgen response, i.e., they cause destruction of the epithelial anlage when exposed to testosterone. Although experimental evidence is not available for other marker genes of the "primary mammary mesenchyme" it appears likely that their expression is also induced by the epithelial gland bud.

Lessons from Gene Deletion Experiments

Several of the genes which are expressed in the embryonic mammary gland have been inactivated by

gene targeting. Such mice provide an opportunity to evaluate the function of these genes in organ development. Tissues from these mice were also used in recombination experiments to ascertain the specific requirement for the genes in either epithelium or mesenchyme.

Surprisingly, mammary glands develop normally in mice deficient for FGF7 (KGF; 21), tenascin-C (22), and syndecan-1 (M. Bernfield, personal communication). The striking localization of PTHrP expression in the peripheral epithelial cells of the bud and of its receptor in the adjoining mesenchymal cells infers a role for this molecule in epithelial signaling. In fact, abolition of this signaling pathway by inactivation of either the peptide or the receptor prevents formation of androgen receptors and outgrowth of the primary sprout at E16 while it does not affect early bud formation (23). Interestingly, the absence of PTHrP signaling has a similar effect in late tooth development, as shown by the failure of teeth to erupt (9).

Mice lacking the transcription factor LEF1 (7) provide the most interesting phenotype with respect to epithelial-mesenchymal interaction in derivatives of the integument. The absence of this gene, which is activated early in all ectodermal rudiments (including hair, whiskers, mammary glands, and teeth) affects the development of all these organs. Though small rudiments of hair and mammary glands are formed, they fail to progress, and whisker development is not even initiated. Tooth development starts normally but is arrested before the formation of the mesenchymal dental papilla (E13.5). The incomplete penetrance of the mutation in hair and mammary gland development might be due to redundant function of the very closely related TCF (T-cell factor) family members. Tissue combinations of wild type and *Lef1* deficient epithelium and mesenchyme carried out in whisker and tooth anlagen have established LEF1 as a chief regulator of epithelium-to-mesenchyme signaling. The requirement for the transcription factor LEF1 is entirely nontissue-autonomous: Whiskers as well as teeth can develop perfectly with both tissue components being mutant, as long as the mesenchyme is transiently associated with *Lef1*-expressing epithelium (8). The epithelial signals downstream of *Lef1* remain to be identified. In this context it should be noted that LEF1 plays a prominent role in WNT-signaling (24).

Tooth development also depends on the activity of the transcription factor MSX1 (25), and preliminary experiments indicate a similar nontissue-autonomous function for this gene, as well (Kratochwil *et al.*, in preparation). In dental mesenchyme (and other instances), MSX1 stimulates transcription of the *Bmp4* gene while being itself activated by BMP-4 (26, 27). These observations suggest that an essential function of specific transcription factors such as MSX1 and LEF1 during early developmental stages is the regulation of tissue-to-tissue signaling. However, these pathways seem to differ from one epithelial organ to another. Thus, *Msx1* is expressed in dental mesenchyme but in the epithelial bud of the developing mammary gland. Mammary development is not affected in *Msx1*-deficient mice, possibly due to redundant function of *Msx2*, since the double knock-out does show a mammary phenotype (R. Maas, personal communication).

Hormone-Induced Tissue Interaction in the E14 Rudiment

To date, the only certain developmental function that can be ascribed to mesenchymal androgen receptors is that they are responsible for the testosterone-induced destruction of the mammary rudiments in E14 male embryos. This sexual dimorphism in the embryonic development of the gland is by no means typical for mammals but represents a peculiarity of the family of some mouse-like rodents. Moreover, since males usually do not need mammary glands, their elimination can be afforded but presumably is of doubtful developmental and evolutionary relevance. Nevertheless, this process has provided important insight into the interplay between classical hormones and tissue interaction.

As mentioned, androgen receptors form in the primary mammary mesenchyme in response to organ-specific signals emitted by the epithelial gland bud. Receptor formation starts by E12, and by E14 the bud in both sexes is surrounded by approximately 3,000 mesenchymal cells, each one possessing about 30,000 binding sites for testosterone (28). The development of hormone-responsiveness through the synthesis of receptors is thus the result of epithelium-to-mesenchyme signaling. In E14 males, fetal androgens cause this mesenchyme to condense around the epithelial bud which eventually is severed from the epidermis and undergoes complete or partial destruction. Testosterone acts only on the mesenchyme since the epithelium has no receptors, and, in an experimental situation, can be taken from an androgen-insensitive mutant (29). Destruction of the epithelium is thus caused by a hormone-induced mesenchymal activity,

and the ligand-dependent transcription factor (androgen receptor) therefore must regulate mesenchyme-to-epithelium signaling. It remains to be resolved whether it causes the output of an apoptotic signal or whether it cuts off the supply of supporting mesenchymal factors. In any case, this process illustrates a specific dependence of hormone action on tissue interaction: first, for the development of hormone responsiveness through the induction of receptor formation, and then, for the execution of the response.

The primary mammary mesenchyme also possesses estrogen receptors which are even functional, as estradiol concentrations below 1 nM block outgrowth of the mammary bud *in vitro* (13). Administration of high doses of estradiol to pregnant mice causes mammary gland and, in particular, nipple malformation (30). Morphologically, this response is distinct from the response to testosterone and rather resembles the consequences of failing PTHrP signaling [inhibited outgrowth of the primary sprout (13)]. Again, the effect of estrogens on epithelial outgrowth must be mediated by the primary mammary mesenchyme.

Epithelial-Mesenchymal Interactions in the Postnatal Mammary Gland

Interactions between epithelium and mesenchyme are also involved in the regulation of ductal outgrowth in the postnatal period. One group of growth factors involved in tissue interaction in the mammary gland that has been uncovered by gene inactivation are the activins and inhibins, members of the transforming growth factor beta (TGFβ) family. Mice lacking the gene encoding the βB subunit of activins and inhibins can neither produce activin B nor inhibin B. These mice have poorly developed mammary glands and are unable to nurse their pups. The mutation inhibits ductal elongation during puberty and alveolar development during pregnancy (Fig. 4; 31). Since activins and inhibins are neuroendocrine regulators and affect the production of reproductive hormones, transplantation and tissue recombination experiments were performed to distinguish between systemic and local effects of the mutation. Glands from wild type mice developed normally when transplanted into mutant mice as did mutant epithelium when it was transplanted into the cleared fat pad of wild type mice. These results show that the attenuated mammary development is not caused by systemic effects and that expression of the βB subunit is dispensable in the mammary epithelium but is required in the mesenchyme. Thus mesenchymal activin and/or inhibin B appear to play a paracrine role in development of both the ducts and alveoli. Further experiments will be required to show whether the production of βB is under hormonal regulation and thus might mediate a hormonal signal that stimulates ductal development.

An important function of epidermal growth factor (EGF) signaling in ductal outgrowth during postnatal mammary development was demonstrated recently. Using recombinations of tissues derived from animals that lack the EGF receptor it was found that the mesenchymal receptor is required for ductal development but its absence does not affect lobulo-alveolar development (32).

Responsiveness to Lactogenic Hormones

The mammary gland reaches its full differentiation as defined by the expression of milk-specific genes only in the adult female after progression through puberty and pregnancy. On the other hand, initiation and determination of the glandular tissue occurs in the embryo as it is reasonable to assume that the entire future gland epithelium derives from the embryonic bud. This observation raises the question of the degree of mammary differentiation of the embryonic epithelium: Is it merely exposure to lactogenic hormones that is required for realization of the differentiated phenotype or does the gland have to pass through further developmental steps in order to acquire full competence to respond to lactogenic stimuli?

We approached these questions by transplanting entire embryonic glands or small fragments of adult mammary glands under the kidney capsule of adult females in various reproductive stages, thereby skipping defined phases of normal gland development. First, glands from E14, newborn and various stages of juvenile to adult mice were directly exposed to a *bona fide* lactogenic environment by transplanting them into lactating females (Fig. 5A). Expression of the milk specific genes β-casein and WAP (whey acidic protein) was analyzed by *in situ* hybridization. In these experiments, all glands from pubertal to pregnant females were found to be capable of activating milk-specific genes. However, neither embryonic rudiments, nor glands from newborn animals were capable of transcribing these genes even after extended periods *in vivo* and despite good overall growth and ductal branching morphogenesis. The critical period for tran-

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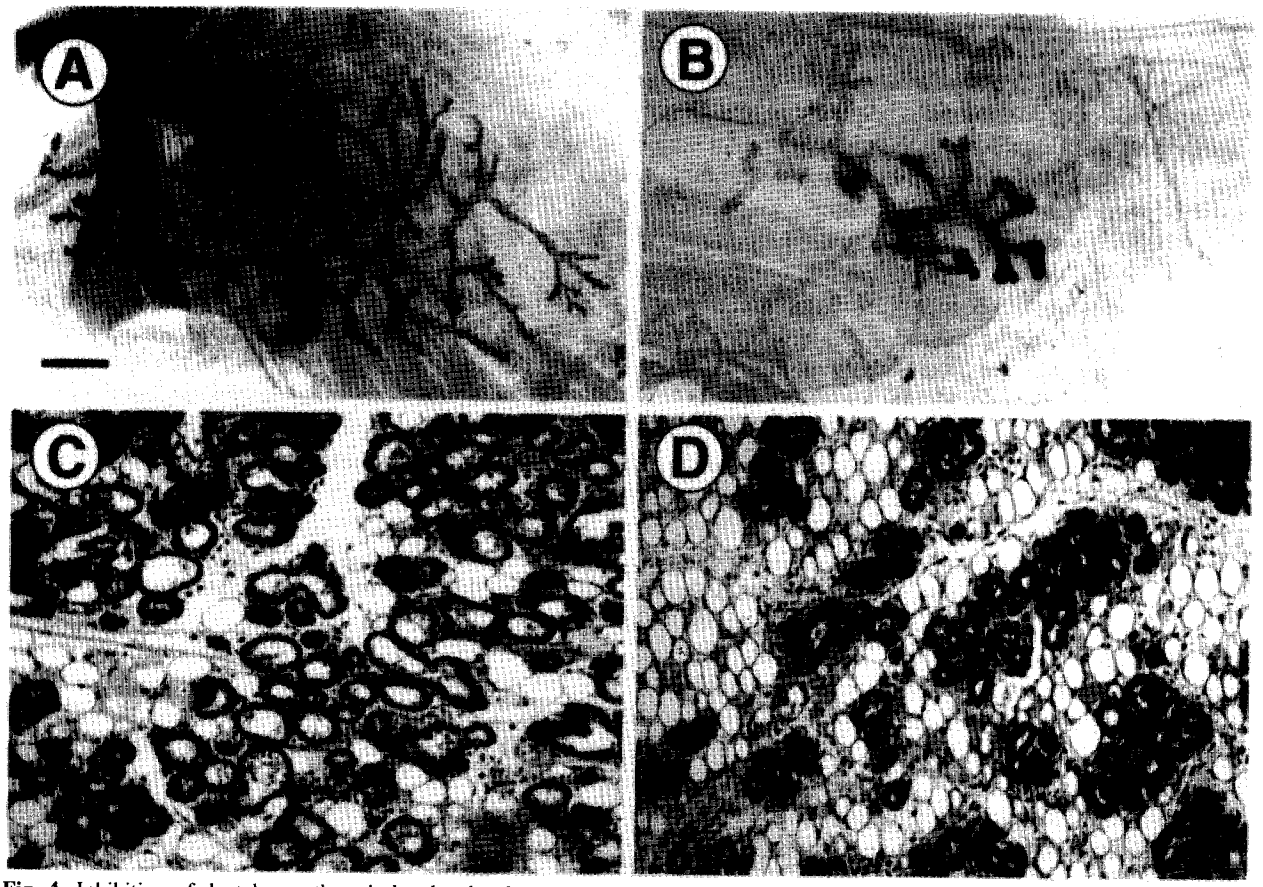

Fig. 4. Inhibition of ductal growth and alveolar development in activin βB-deficient mammary glands. (A) Whole mount of second thoracic gland of 7 week old wild type mouse. (B) Second thoracic gland of activin βB-deficient littermate. (C) Section of wild type mammary gland on day 18 of pregnancy stained with haematoxyline and eosin. (D) Section of activin βB-deficient mammary gland of the same stage. Note the sparse filling of the fat pad with mammary epithelium and the small size of alveoli. Bar: 500 μm (A and B), 80 μm (C and D).

sition from incompetence to lactogenic competence occurred around four weeks after birth, i.e., very early in puberty. These results argue against a strict requirement for the gland to pass through pregnancy in order to express milk protein genes; rather an essential step towards the capacity for full differentiation takes place with the onset of puberty. These results differ from the aforementioned findings by Cunha who demonstrated activation of milk protein synthesis when embryonic epidermis was associated with primary mammary mesenchyme and transplanted into pituitary grafted hosts (18). These experiments differ in many aspects and are hard to compare. Among others, differences in the timing of exposure to and the levels of prolactin and progesterone may explain this divergence.

While glands from E15 embryos failed to transcribe the β-casein and WAP genes when transferred directly into lactating hosts, these genes were expressed if the same rudiments were transplanted into the kidney capsule of mature virgins and then exposed to the hormones of pregnancy and early lactation within their hosts (Fig. 5B). In order to narrow down the time required in the host before actual lactation, we transplanted E15 glands into hosts at different stages of pregnancy and found that one day in the pregnant host was sufficient for differentiation: Gland rudiments transplanted the day before parturition were capable of milk gene expression when examined 8 days later. There are major differences in the hormonal milieu between pregnancy and lactation in the levels of both progesterone and estrogen and there is often a postpartum surge of progesterone related to an early ovulation event. In order to mimic this event we injected lactating females receiving embryonic mammary gland transplants with progesterone at surgery. Under these conditions, transcription of milk protein genes took

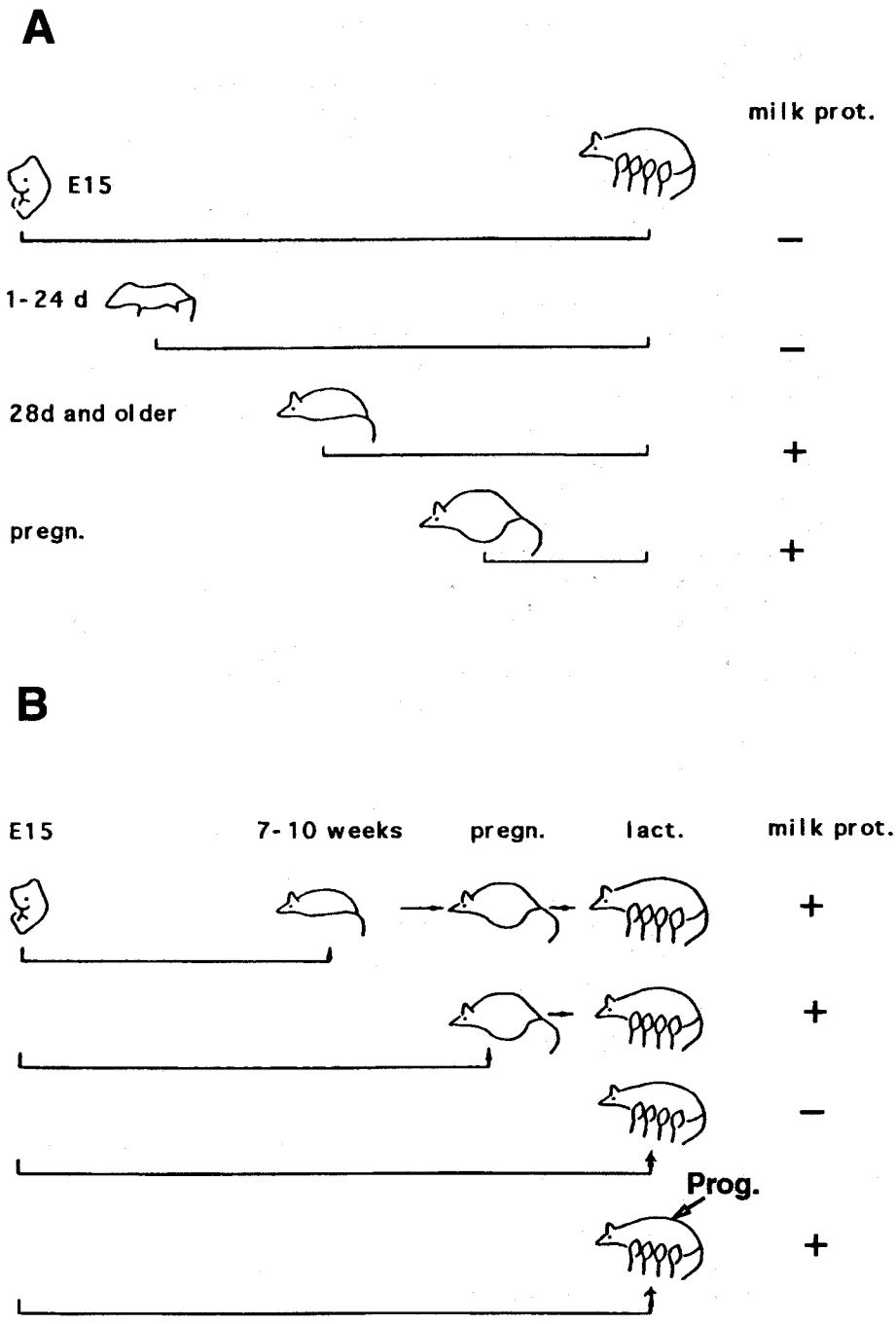

Fig. 5. Schematic presentation of transplantation experiments. (A) Mammary tissues from different developmental stages were transplanted into lactating hosts. Expression of milk proteins was observed only when the transplanted tissue was derived from postpubertal donors. (B) Transplanted embryonic glands activated milk protein genes when transplanted into virgin or pregnant mice. Embryonic transplants into lactating hosts expressed milk proteins only when the recipients were treated with progesterone.

place, clearly indicating that this hormone supplement was sufficient in the presence of high levels of prolactin to activate differentiation specific genes in the embryonic epithelium.

These results should not be interpreted to indicate that a combination of progesterone and prolactin can directly induce lactation in fetal glands. Expression of milk-specific genes in the grafts required a minimum of 7 days during which the tissues proliferated and underwent active morphogenesis. It also should be noted that only alveolar morphology and synthesis of β-casein and WAP mRNA were assessed in these experiments while we do not know whether the transplants are able to synthesize lactose and secrete milk. However, the experiments do suggest an obligatory role for progesterone in the acquisition of lactogenic responsiveness. Possibly, the low progesterone levels of early pubertal females fulfill this function, and the gland does not necessarily have to pass through pregnancy. Experiments analyzing the sequence of events during this condensed maturation of the gland are likely to shed important light on the mechanisms involved in full differentiation of the mammary epithelium.

CONCLUSIONS

Several discrete steps can be defined in the process of mammary gland development. At its onset, a subset of cells in the primitive epidermis undergoes morphologic and biosynthetic diversification while inducing developmental changes in the most adjacent mesenchyme. Thereby, distinct mammary epithelial and mesenchymal cell populations are established that can be characterized by the expression of specific genes. At this stage each tissue is apparently capable of inducing an undetermined partner to participate in mammary development in experimental association. Reciprocal interactions and mutual dependence of the two tissues continue as demonstrated by the testosterone response and the inhibition of ductal outgrowth in the absence of PTHrP signaling. Interactions between mammary epithelium and its secondary stroma, the fat pad, lead to further proliferation and morphogenesis and the establishment of a rudimentary epithelial system at birth. Even though a complex maturation process set in motion by endocrine influences is required in the intact animal for complete development of a functional gland, the embryonic mammary gland has the potential to respond to lactogenic hormones and thus appears to be fully determined.

A better understanding of the mechanisms of tissue interaction will derive from the study of other gene deletion models. Several previously unknown signaling molecules have already been identified by this technology. The function of these molecules as potential mediators of tissue interactions can then be ascertained by direct application, either in solution or by local release from beads. The analysis of localized gene expression during critical periods of development by *in situ* hybridization or immunohistochemistry will provide further clues to their participation in short range signaling.

Work on many developing systems has revealed that vertebrate organogenesis is mediated by the exchange of signals belonging to a limited number of superfamilies. It is therefore not surprising that the derivatives of the skin, i.e., feathers, hair, whiskers, mammary gland, and tooth, appear to share some of these signaling pathways. It is more remarkable that both tooth and mammary development depend on PTHrP signaling, and that both express PPT-A, as these molecules do not belong to the illustrious and almost ubiquitously acting signal families. In view of the much greater variety of transcription factors, it is puzzling that some of them are also shared by various skin derivatives though they are not necessarily expressed in the same tissue compartment (as *Msx1* in tooth and mammary gland). Of particular interest among them is *Lef1* which might be an ectodermal marker gene for the initiation of appendages. *Lef1* is not only expressed in all these sites, its absence also affects the development of all these organs. Elucidation of the functional hierarchies linking transcription factors to signaling and signal reception would seem to be one of the most important steps in understanding organogenetic tissue interactions.

REFERENCES

1. A. Propper and L. Gomot (1967). Interactions tissulaires au cours de l'organogenèse de la glande mammaire de l'embryon de lapin. *Compt. Rend. Acad. Sci. Paris* **264**:2573–2575.
2. N.K. Wessells (1965). Morphology and proliferation during early feather development. *Dev. Biol.* **12**:131–153.
3. N.K. Wessells and K.D. Roessner (1965). Nonproliferation in dermal condensations of mouse vibrissae and pelage hairs. *Dev. Biol.* **12**:419–433.
4. K. Kratochwil (1969). Organ specificity in mesenchymal induction demonstrated in the embryonic development of the mammary gland of the mouse. *Dev. Biol.* **20**:46–71.

5. T. Sakakura, Y. Sakagami, and Y. Nishizuka (1979). Persistence of responsiveness of adult mouse mammary gland to induction by embryonic mesenchyme. *Dev. Biol.* **72**:201–210.
6. T. Sakakura, I. Kusano, M. Kusakabe, Y. Inaguma, and Y. Nishizuka (1987). Biology of mammary fat pad in fetal mouse: Capacity to support development of various fetal epithelia *in vivo*. *Development* **100**:421–430.
7. C. van Genderen, R.M. Okamura, I. Fariñas, R. G. Quo, T.G. Parslow, L. Bruhn, and R. Grosschedl (1994). Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in *Lef-1* deficient mice. *Genes Dev.* **8**:2691–2703.
8. K. Kratochwil, M. Dull, I. Fariñas, J. Galceran and R. Grosschedl (1996). *Lef1* expression is activated by *Bmp-4* and regulates inductive tissue interactions in tooth and hair development. *Genes Dev.* **10**:1382–1394.
9. J.J. Wysolmerski, W.M. Philbrick, M.E. Dunbar, B. Lanske, H. Kronenberg, A. Karaplis, and A.E. Broadus (1998). Rescue of parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. *Development* **125**:1285–1294.
10. D.J. Phippard, S.J. Weber-Hall, P.T. Sharpe, M.S. Naylor, H. Jayatalake, R. Maas, I. Woo, D. Roberts-Clark, P.H. Francis-West, Y.H. Liu, R. Maxson, R.E. Hill, and T.C. Dale (1996). Regulation of *Msx-1, Msx-2, Bmp-2*, and *Bmp-4* during foetal and postnatal mammary gland development. *Development* **122**:2729–2737.
11. D. Houzelstein, A. Cohen, M.E. Buckingham and B. Robert. (1997). Insertional mutation of the mouse *Msx1* homeobox gene by an *nlacZ* reporter gene. *Mech. Dev.* **65**:123–133.
12. B. Heuberger, I. Fitzka, G. Wasner, and K. Kratochwil (1982). Induction of androgen receptor formation by epithelium-mesenchyme interaction in embryonic mouse mammary gland. *Proc. Natl. Acad. Sci. U.S.A.* **79**:2957–2961.
13. Kratochwil, K. (1982). The importance of epithelial-stromal interaction in mammary gland development. In M. A. Rich, J.C. Hager, and J. Taylor-Papadimitriou, (eds.), *Breast Cancer: Origins, Detection, and Treatment*, Martinus Nijhoff Publishing, Boston: pp. 1–12.
14. M. Weil, A. Itin, and E. Keshet (1995). A role for mesenchyme-derived tachykinins in tooth and mammary gland morphogenesis. *Development* **121**:2419–2428.
15. R. Chiquet-Ehrisman, E.J. Mackie, C.A. Pearson, and T. Sakakura (1986). Tenascin: An extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell* **10**:131–139.
16. G.R. Cunha and Y.K. Hom (1996). Role of mesenchymal-epithelial interactions in mammary gland development. *J. Mam. Gland Biol. Neoplasia* **1**:21–35.
17. I. Thesleff and P. Sharpe (1997). Signaling networks regulating dental development. *Mech. Dev.* **67**:111–123.
18. G.R. Cunha, P. Young, K. Christov, R. Guzman, S. Nandi, F. Talamantes, and G. Thordarson (1995). Mammary phenotypic expression induced in epidermal cells by embryonic mammary mesenchyme. *Acta Anat.* **152**:195–204.
19. C. Rodriguez-Esteban, J.W.R. Schwabe, J. De La Peña, B. Foys, B. Eshelman, and J.C. Izpisúa Belmonte (1997). Radical fringe positions the apical ectodermal ridge at the dorsoventral boundary of the vertebrate limb. *Nature* **386**:360–366.
20. E. Laufer, R. Dahn, O.E. Orozco, C-Y Yeo, J. Pisenti, D. Henriques, U.K. Abbott, J.F. Fallon, and C. Tabin (1997). Expression of radical fringe in limb-bud ectoderm regulates apical ectodermal ridge formation. *Nature* **386**:366–373.
21. L. Guo, L. Degenstein, and E. Fuchs (1996). Keratinocyte growth factor is required for hair development but not for wound healing. *Genes Dev.* **10**:165–175.
22. Y. Saga, T. Yagi, Y. Ikawa, T. Sakakura, and S. Aizawa (1992). Mice develop normally without tenascin. *Genes Dev.* **6**:1812–1831.
23. M.E. Dunbar and J.J. Wysolmerski (1999). Parathyroid hormone-related protein: A developmental regulatory molecule necessary for mammary gland development. *J. Mam. Gland Biol. Neoplasia* **4**(1):XX–XX.
24. H.C. Clevers and M. van de Wetering (1997). TCF/LEF factors earn their wings. *Trends Genet.* **13**:485–489.
25. I. Satokata and R. Maas (1994). *Msx1* deficient mice exhibit cleft-palate and abnormalities of craniofacial and tooth development. *Nature Genet.* **6**:348–356.
26. Y. Chen, M. Bei, I. Woo, I. Satokata, and R. Maas (1996). *Msx1* controls inductive signaling in mammalian tooth morphogenesis. *Development* **122**:3035–3044.
27. S. Vainio, I. Karavanova, A. Jowett, and I. Thesleff (1993). Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell* **75**:45–58.
28. G. Wasner, I. Hennerman, and K. Kratochwil (1983). Ontogeny of mesenchymal androgen receptors in the embyonic mouse mammary gland. *Endocrinology* **113**:1771–1780.
29. K. Kratochwil and P. Schwartz (1976). Tissue interactions in androgen response of embryonic mammary rudiment of mouse: Identification of target tissue for testosterone. *Proc. Natl. Acad. Sci. U.S.A.* **73**:4041–4044.
30. A. Raynaud, (1961). Morphogenesis of the mammary gland. In S.K. Kon, and A.T. Cowie, (eds.), *The Mammary Gland and Its Secretions*, Academic Press, New York pp. 3–46.
31. G.W. Robinson and L. Hennighausen (1997). Inhibins and activins regulate mammary epithelial cell differentiation through mesenchymal-epithelial interactions. *Development* **124**:2701–2708.
32. J.F. Wiesen, P. Young, Z. Werb, and G.R. Cunha. (1998). Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development. *Development* **126**:335–344.